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Lymphocyte cytotoxicity for autologous human hepatocytes in alcoholic liver disease

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SUMMARY

Alcoholic liver disease has been shown to progress even after cessation of ethanol intake and the involvement of an immunological mechanism has been suggested. To study whether lymphocyte cytotoxicity for autologous human hepatocytes is involved in the pathogenic process of alcoholic liver disease, hepatocytes (target cells) obtained by a needle liver biopsy from 36 patients with alcoholic liver disease were isolated by enzymatic digestion and incubated with autologous peripheral lymphocytes (effector cells). Using a microcytotoxicity assay, a cytotoxic effect was observed in patients with active cirrhosis or alcoholic hepatitis, but not in those with inactive cirrhosis, hepatic fibrosis or fatty liver. When lymphocytes were separated into T cell enriched and non-T cell enriched fractions, this cytotoxic effect was significantly greater with the non-T cell enriched lymphocyte fraction than with the T cell enriched fraction. The addition of aggregated IgG reduced the cytotoxic effect of the lymphocytes. These results suggested that antibody-dependent cell-mediated cytotoxicity may be of pathogenic importance in alcoholic liver disease.

Keywords alcoholic liver disease lymphocyte cytotoxicity autologous human hepatocytes ADCC

INTRODUCTION

Since alcohol (ethanol) *per se* is a hepatotoxin, hepatic lesions observed in alcoholic liver disease can be explained as a direct effect of alcohol (Lieber, 1966, 1975, 1981). Nevertheless, an immunological process has been suggested to play some role in the pathogenesis of alcoholic liver disease, because only a minority of alcoholics develop cirrhosis (Klatskin, 1961), and liver injury may continue even after cessation of alcohol intake (Galambos, 1972). Indeed, lymphocytes of patients with alcoholic hepatitis are reportedly cytotoxic for Chang liver cells (Kakumu *et al.*, 1977), rabbit hepatocytes (Cochrane *et al.*, 1977), and for the patients' own liver cells (Kakumu *et al.*, 1977). Theoretically, lymphocyte cytotoxicity requires the sharing of HLA compatibility restriction between effector and target cells; therefore, an autologous system is the best experimental model to provide an assay for lymphocyte cytotoxicity. In experiments using isolated human hepatocytes, however, when a ^{51}Cr release assay was utilized for the lymphocyte-mediated cytotoxicity test (Kakumu *et al.*, 1977), spontaneous ^{51}Cr release from the cultured hepatocytes was found to be very high.

In the present study, we tested to see whether lymphocyte cytotoxicity for autologous hepatocytes was involved in the pathogenic process in patients with alcoholic liver disease. We used a microcytotoxicity assay, in which freshly isolated human hepatocytes obtained by needle biopsy

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were cultured with autologous lymphocytes and the remaining hepatocytes were then counted. The possible immunological mechanism involved in this process was also investigated.

MATERIALS AND METHODS

Patients. Thirty-six patients with alcoholic liver disease (all males) were included in this study. All of the patients had a history of drinking more than 80 g of alcohol per day more than 10 years. None of them had a history of blood transfusion or drug abuse, and none had positive HBs antigen or HBc antibody in his serum. Liver biopsies were performed within 2 weeks of admission and the patients classified, according to their histological findings, into five groups: 12 cases of active cirrhosis, five of inactive cirrhosis, three of alcoholic hepatitis, nine of hepatic fibrosis and seven of fatty liver. Active cirrhosis was differentiated from inactive cirrhosis by the presence of piecemeal necrosis associated with extensive inflammation. The diagnosis of alcoholic hepatitis was in accord with the Fogarty nomenclature (Leevy & Tygstrup, 1976). Patients with alcoholic hepatitis superimposed on an underlying cirrhosis were not included in the present study. The diagnosis of hepatic fibrosis was established by the presence of the following abnormalities (Takada *et al.*, 1982): (1) pericellular creeping fibrosis mainly in the centrolobular area and sometimes in the portal area, (2) relatively scanty cellular inflammation in the portal triads with or without fatty metamorphosis and (3) absence of the features of alcoholic hepatitis, chronic hepatitis and cirrhosis.

Seven patients who had no history of alcohol intake and whose liver samples were obtained during operations for peptic ulcer or cholelithiasis served as controls.

Isolation of hepatocytes. Isolation of hepatocytes was performed in accordance with the method of Bellemann *et al.* (1977) with slight modifications. In brief, the liver biopsy specimen was cut into small pieces in phosphate-buffered saline (pH 7·2) at 4°C, and was incubated in Hanks' balanced salt solution (HBSS) containing 0·05% collagenase for 30 min at 37°C. The pellet was filtered through four layers of gauze and was centrifuged at 50*g* for 3 min. After a wash with HBSS, the sediment was incubated in HBSS containing 0·015% trypsin and 0·01% DNAase for 2 min in order to remove trypan blue stained cells. After another wash, the pellet composed of isolated hepatocytes was resuspended in Eagle's medium including 10% fetal calf serum (FCS), 10^{-6}M insulin, penicillin (100 iu/ml) and streptomycin (100 μg/ml). Cell viability, determined by the trypan blue exclusion test, was 95·2 $\pm$ 2·3% (mean $\pm$ s.e.). When examined under a scanning electron microscope, these cells showed intact microvilli on the surface (Fig. 1).

Isolation of peripheral blood lymphocytes. Peripheral blood lymphocytes were obtained from 20 ml of heparinized peripheral venous blood by centrifugation over a Ficoll/Conrey density gradient (Böyum, 1968). This lymphocyte pellet was incubated in a FCS coated plastic petri dish for 1 h to remove macrophages (Kumagai *et al.*, 1979). These lymphocytes contained less than 4% macrophages, as determined by peroxidase staining. When indicated, the lymphocytes were further separated into T cell enriched and non-T cell enriched fractions, using the sheep erythrocyte sedimentation method (Yata *et al.*, 1973). The T cell enriched fraction contained less than 7·8% surface immunoglobulin positive cells, and the non-T cell enriched fraction contained less than 9·8% sheep erythrocyte rosette forming cells.

Cytotoxicity test. A cytotoxicity test was carried out a microcytotoxicity assay (Takasugi & Klein, 1970). Three hundred hepatocytes were seeded into each well of a microplate (Falcon 3034) and were incubated for 24 h under 95% O_2 and 5% CO_2. After removing non-viable cells by inverting the plate and washing gently with phosphate-buffered saline, peripheral blood lymphocytes were added to each well with a hepatocytes/lymphocytes ratio of 1/20. The other 10 wells were filled with medium alone as controls. When indicated, lymphocytes which were treated with aggregated IgG to a final concentration of 50 μg/ml were used in order to test the blocking effect of the Fc receptor of lymphocytes on the cytotoxicity test. The microplate thus treated was incubated for 48 h under 95% O_2 and 5% CO_2 at 37°C. After the incubation period, the plate was inverted for 2 h to allow detachment of non-viable hepatocytes and to clear the added lymphocytes. The remaining

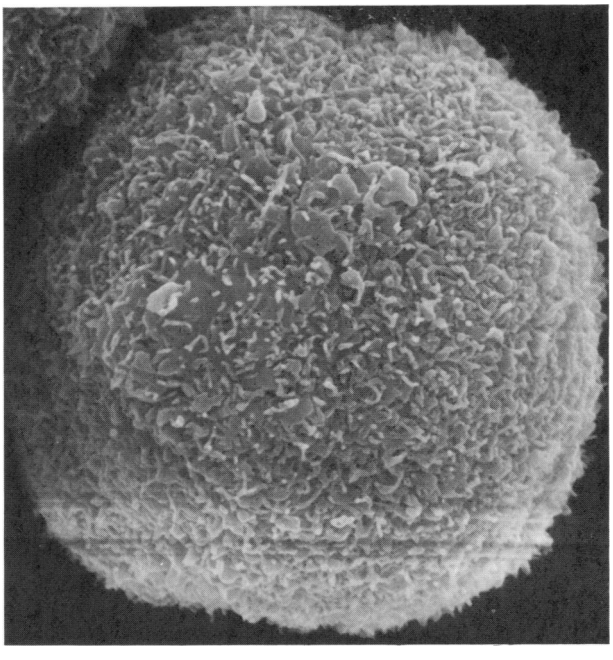

Fig. 1. An isolated hepatocyte examined under scanning electron microscope. The microvilli on the surface seem to be intact.

hepatocytes were counted and the percentage cytotoxicity was calculated according to the following formula:

$$\% \text{ cytotoxicity} = 100 - \frac{\text{hepatocytes in experimental wells (10 wells)}}{\text{hepatocytes in control wells (10 wells)}} \times 100.$$

The upper limit of the normal range for cytotoxicity (9·9%) was taken as two standard deviations above the mean value (4·1%) obtained in seven normal controls.

Statistical analysis. Statistical significance of the results was assessed by the paired or unpaired Student's *t*-test (Snedecor & Cochrane, 1967) as indicated.

RESULTS

Significant lymphocyte-mediated cytotoxicity for autologous hepatocytes was demonstrated in 10 of 12 patients with active cirrhosis (83%), and in all three patients with alcoholic hepatitis (100%). By contrast, only a few patients with inactive cirrhosis (20%), hepatic fibrosis (22%) or fatty liver (14%) showed significant cytotoxicity (Fig. 2). Thus the mean ($\pm$s.e.) value for % cytotoxicity was found to be higher in patients with active cirrhosis than in normal controls ($18{\cdot}2 \pm 9{\cdot}1\%$ *vs* $4{\cdot}1 \pm 2{\cdot}9\%$, $P < 0{\cdot}001$, unpaired Student's *t*-test).

When autologous hepatocytes were cultured with T cell enriched fractions, the cytoxic effect was considerably reduced to $11{\cdot}0 \pm 6{\cdot}2\%$ from the $20{\cdot}8 \pm 7{\cdot}0\%$ observed with non-fractionated lymphocytes ($P < 0{\cdot}001$, paired *t*-test). By contrast, there appeared to be no significant difference between non-T cell enriched preparations and non-fractionated lymphocytes with respect to the cytotoxic reaction (Fig. 3). Although all cases except one showed higher cytotoxicity with non-T enriched lymphocyte fraction than with T cell enriched lymphocyte fraction, T cell enriched fraction in seven cases still showed significant cytotoxicity. Furthermore, in one case, cytotoxicity appeared to be mediated by T lymphocytes only.

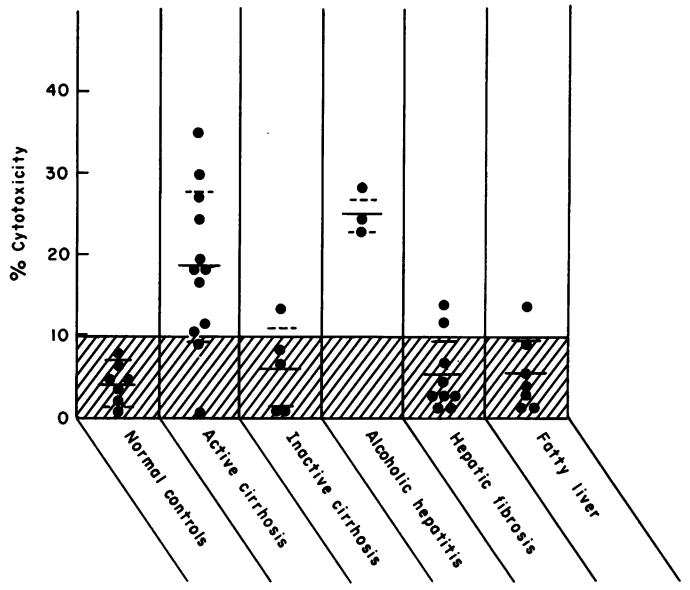

Fig. 2. Cytotoxicity in autologous hepatocytes induced by non-fractionated lymphocytes. The cytotoxicity in patients with active cirrhosis was significantly higher than in normal controls ($P < 0{\cdot}001$, Statistical significance was assessed by Student's t-test.).

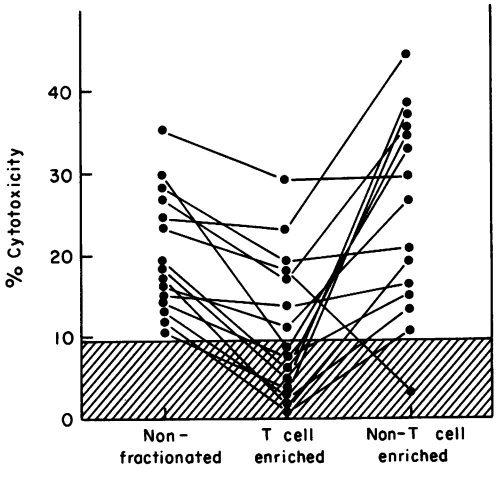

Fig. 3. The comparison of cytotoxicity induced by T cell and non-T cell enriched lymphocyte fractions. Non-T cell enriched fraction showed significantly higher cytotoxicity than T cell enriched fraction ($P < 0{\cdot}001$, Statistical significance was assessed by paired t-test.).

In five patients who showed a significant cytotoxic effect for autologous hepatocytes, the addition of 50 μg/ml of aggregated IgG to non-fractionated lymphocytes reduced the mean ($\pm$s.e.) percentage of cytotoxicity from $24{\cdot}3 \pm 5{\cdot}8\%$ to $11{\cdot}6 \pm 4{\cdot}6\%$ (Fig. 4); this reduction in cytotoxicity was statistically significant ($P < 0{\cdot}05$, paired t-test). In three cases, T cell enriched and non-T cell enriched lymphocytes were separately treated with aggregated IgG. The addition of aggregated IgG to T cell enriched fraction did not change the mean ($\pm$s.e.) percentage of cytotoxicity (from $14{\cdot}6 \pm 10{\cdot}1\%$ to $13{\cdot}5 \pm 7{\cdot}7\%$). By contrast, cytotoxic effect was markedly reduced from $33{\cdot}0 \pm 2{\cdot}9\%$ to $12{\cdot}3 \pm 1{\cdot}5\%$ when non-T cell enriched fraction was treated with aggregated IgG.

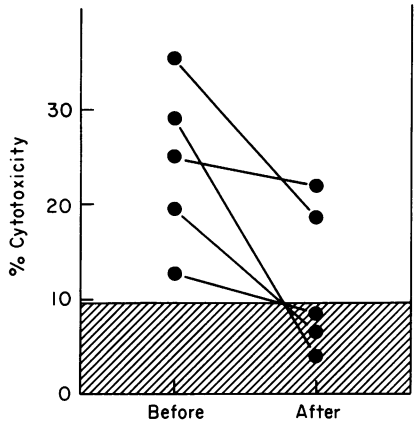

Fig. 4. The comparison of cytotoxicity before and after the addition of aggregated IgG. The addition of 50 μg/ml of aggregated IgG to non-fractionated lymphocytes significantly reduced the percentage of cytotoxicity ($P < 0{\cdot}05$, paired t-test).

DISCUSSION

In the present study, lymphocyte cytotoxicity for autologous hepatocytes was clearly shown in alcoholic patients with active cirrhosis or alcoholic hepatitis. In addition, this cytotoxic effect was found to be mediated considerably with non-T lymphocytes, and non-T lymphocyte-mediated cytotoxicity was markedly blocked by the addition of aggregated IgG. These results suggest that antibody-dependent cell-mediated cytotoxicity which is responsible for the cytotoxic effect observed in the present study may be of pathogenic importance in alcoholic liver injury.

It has been reported already that in patients with alcoholic hepatitis, peripheral lymphocytes are cytotoxic toward a variety of target cells; in those studies, however, only an experiment reported by Kakumu *et al.* (1977) used the patients' own liver cells as target cells for the demonstration of the lymphocyte-mediated cytotoxic effect. In that study, a ^{51}Cr releasing assay was employed for the cytotoxic test, but spontaneous release of ^{51}Cr from the cultured hepatocytes was found to be very high. Thus the ^{51}Cr releasing assay used appeared to lack sensitivity with respect to the determination of lymphocyte-mediated cytotoxic effect for autologous hepatocytes. In addition, they did not determine which lymphocyte fraction induced the cytotoxic effect. Their results need to be confirmed with a more sensitive method, and the detailed immunological mechanism remains to be studied.

In the present study, we used isolated autologous hepatocytes as target cells, and a microcytotoxicity assay was employed for the cytotoxic test. The system with autologous hepatocytes has at least two advantages over using hepatocytes of other species. Firstly, the effector lymphocytes and target hepatocytes are fully HLA compatible; this is an important requirement for optimal T cell cytotoxicity (Zinkernagel, 1978). Even though we used this autologous system, the cells that showed the cytotoxic effect were non-T lymphocytes. Secondly, various expressions of surface antigens of the hepatocytes are preserved. Recently, MacSween *et al.* (1981) reported the existence of antibodies to alcohol altered hepatocytes in the sera of patients with alcoholic liver disease. These neoantigens of hepatocytes induced by alcohol are thought to be preserved on the isolated human hepatocytes.

In experimental models of alcoholic liver disease in baboons, Paronetto & Lieber (1976) and Lue *et al.* (1981) showed lymphocyte cytotoxicity for autologous hepatocytes even in the stage of fatty liver. In the present study, however, lymphocyte cytotoxicity for autologous hepatocytes was found only in the patients with active cirrhosis and alcoholic hepatitis. The reason for this discrepancy is not clear, but it could be suggestive of a difference in immunological response between the baboon and man.

Antibody-dependent cell-mediated cytotoxicity has been suggested to take part in liver cell

damage in alcoholic liver disease. Indeed, using isolated rabbit hepatocytes as target cells, Cochrane *et al.* (1977) showed that lymphocyte cytotoxicity for hepatocytes was non-T cell-mediated and was blocked by the addition of liver specific lipoprotein (LSP). They also reported the existence of anti-LSP antibody in the sera of patients with alcoholic liver disease. Since there is a close association between the presence of the anti-LSP antibody and the findings on liver biopsy of lymphocytic infiltrates in the portal tracts together with piecemeal necrosis (Perperas *et al.*, 1981), they have suggested that an autoimmune response to LSP might be important in the progression of alcoholic hepatitis to chronic liver injury.

In the present study, however, T cell enriched lymphocyte fraction showed still significant cytotoxicity in seven cases (Fig. 3). Furthermore, in one case, cytotoxicity was mediated only by T cell enriched fraction. These results suggest that either cytotoxic T cells, reacting with a neoantigen on the target cell surface, or natural killer cells may also play some role in lysis of autologous hepatocytes. It has been shown that natural killer cells bear low affinity receptors for sheep erythrocytes (West *et al.*, 1977, 1978).

Nevertheless, the present observations that the cytotoxic effects were mediated mostly by non-T lymphocytes and blocked by the addition of aggregated IgG gives further support to the significance of antibody-dependent cell-mediated cytotoxicity responsible for the hepatocyte cytotoxicity in alcoholic liver disease.

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